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Induction of terminal differentiation and apoptosis in human colonic carcinoma cells by brefeldin A, a drug affecting ganglioside biosynthesis

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Abstract An appreciable increase in G_{M3} with a concomitant decrease in some neolacto-series gangliosides was observed during differentiation of human colonic carcinoma HCT 116 cells induced by a differentiating agent. When the cells were treated with brefeldin A (BFA), a striking increase in de novo biosynthesis of G_{M3} and a decrease in biosynthesis of neolacto-series gangliosides were observed after 6 h. Clear morphological changes to differentiated epithelial cells and an arrest of cells in the G_0/G_1 phase of the cell cycle were observed after 1 day of treatment. Then the cells were led to apoptosis. This activity was not affected by forskolin, which antagonizes the effects of BFA on protein transport and the Golgi apparatus. These results suggest that the differentiation-inducing activity of BFA might be due to its modulatory effect on ganglioside biosynthesis, and that a specific change in ganglioside pattern is an essential prerequisite for induction of differentiation, providing a novel target for differentiation therapy of cancer.

Key words: Brefeldin A; Ganglioside; Differentiation; Apoptosis; Human colonic carcinoma cell

1. Introduction

Glycosphingolipids (GSLs) are amphiphilic membrane components. Gangliosides, GSLs containing sialic acid, are considered to be involved in regulation of cell growth and differentiation [1]. We have shown that particular gangliosides increase, depending on differentiation direction, during differentiation of human myelogenous leukemia cells induced by differentiating agents [2], and that such gangliosides themselves induce differentiation in leukemia cells along the same direction as their characteristic increases are observed [3,4]. These results have led to the hypothesis that specific changes of ganglioside pattern promote or induce terminal differentiation of leukemia cells and that defects in ganglioside expression might be one reason for differentiation arrest of leukemia cells at an immature stage.

We examined whether the hypothesis is applicable to other malignant tumor cells using the human colonic carcinoma cell line HCT 116 and found that human epithelial carcinoma cells can also be induced to differentiate into terminal differentiated cells by remodeling of the ganglioside pattern.

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Abbreviations: BFA, brefeldin A; NaBT, sodium butyrate; HPTLC, high-performance thin-layer chromatography; Designation of ganglio-series gangliosides is according to Svennerholm [27] and designation of other gangliosides is according to IUPAC nomenclature [28]

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was obtained from Iwaki Glass Co., Tokyo, Japan. Fetal bovine serum (FBS) was obtained from HyClone, Logan, UT, USA. Tissue culture plasticware was purchased from Becton Dickinson Labware, Lincoln Park, NJ, USA. L-[3-^{14}C]Serine was purchased from Amersham, UK. [4-^{14}C]-Cholesterol was purchased from NEN, Boston, MA, USA. Silica gel 60 HPTLC plates were obtained from Merck, Darmstadt, Germany. All other reagents were of analytical grade and were obtained from Sigma, St. Louis, MO, USA. The human colonic carcinoma cell line HCT 116 [5] was obtained from the American Type Culture Collection.

2.2. Cell culture

HCT 116 cells were grown in Falcon 3082 tissue culture flasks in DMEM containing 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were counted with a hemocytometer, and viability of cells was assessed by a dye exclusion test with erythrosin B. Treatment of cells with sodium butyrate (NaBT) was carried out by culturing them in the aforementioned medium containing 1 mM NaBT. Brefeldin A (BFA) and forskolin were dissolved in 99.5% ethanol at a concentration of 0.1 mg/ml (0.36 mM) and 50 mM respectively, and added into the growth medium such that the final ethanol concentration was 0.1%. Because this concentration of ethanol had no effect on the cells, an ethanol control was not included in this study.

2.3. Analysis of gangliosides

Total lipids were extracted sequentially from cells with the lower phase of isopropyl alcohol/hexane/water (55:25:20, v/v/v), chloroform/methanol (1:1, v/v), and chloroform/methanol (2:1, v/v) [6]. In the radiolabeling study, [4-^{14}C]cholesterol was used as an internal standard. Gangliosides were prepared by Folch's partition and DEAE-Sephadex A-25 chromatography from total lipids [7] and resolved by thin-layer chromatography on silica gel 60 HPTLC plates with a solvent system composed of chloroform/methanol/0.5% $CaCl_2$ (50:40:10, v/v/v). Measurement was done by densitometric scanning [2].

2.4. De novo biosynthesis of gangliosides

HCT 116 cells were cultured in the presence of 300 nCi/ml L-[3-^{14}C]serine for various times [8]. After labeling, gangliosides were prepared and resolved as described above. The radioactivity of each band was determined using a BAS 2000 imaging analyzer (Fuji Film, Tokyo, Japan).

2.5. Cell cycle analysis

DNA contents of cells were measured using the propidium iodide (PI) staining method and a flow cytometer [9]. The harvested cells (2×10^6) were fixed in ice-cold 70% ethanol for 30 min. After washing with phosphate-buffered saline, cells were treated with 200 μl of 0.5 mg/ml DNase-free RNase A at 37°C for 20 min. Then the cells were stained with 500 μl of 0.05 mg/ml PI at 4°C for 10 min, and DNA content was analyzed with an Epics Elite flow cytometer (Coulter, Hialeah, FL, USA).

2.6. Analysis of DNA fragmentation

DNA fragmentation was analyzed using agarose gel electrophoresis [10]. Cells (1×10^6) were incubated at 50°C for 90 min in a solution

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containing 10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 10 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K. Then DNase-free RNase A was added to a final concentration of 20 μg/ml and incubated at 37°C for 1 h. DNA was prepared by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitation with ethanol. DNA (2 μg) was loaded and electrophoresed in a 2.0% agarose gel containing 0.1 mg/ml ethidium bromide. DNA was made visible under UV light.

3. Results

3.1. Differentiation-related changes in ganglioside pattern of HCT 116 cells

NaBT has been reported to induce HCT 116 cells to differentiate [11]. When we treated HCT 116 cells with 1 mM NaBT, gross morphological changes, including cell enlargement and flattening, were observed after 2 days of treatment with growth suppression (Fig. 1A). There was no piling up of treated cells in the culture. However, prominent nucleoli remained in many NaBT-treated cells and nuclear condensation was inadequate, suggesting that NaBT-treated cells are not necessarily fully differentiated cells.

As shown in Fig. 1B, gangliosides of HCT 116 cells consisted of at least 13 molecular species, including ganglio-series gangliosides G_{M3} (a doublet, band 1) and G_{M2} (a doublet, band 2). The R_f values of gangliosides designated doublet band 3, band 4, and band 6 were similar to those of the sialosyl α2→6-lacto-*neo*tetraosylceramide ($IV^6NeuAcnLc_4$) doublet, sialosyl α2→3-lacto-*nor*hexaosylceramide ($VI^3NeuAcnLc_6$), and sialosyl α2→6-lacto-*nor*hexaosylceramide ($VI^6NeuAcnLc_6$) of human erythrocytes respectively. The presence of the $IV^6NeuAcnLc_4$ doublet and $VI^6NeuAcnLc_6$ in human colonic adenocarcinoma has been reported [7,12]. During NaBT-induced differentiation of HCT 116 cells, G_{M3}, G_{M2}, and unidentified bands 5 and 7 increased appreciably while ganglioside corresponding to $VI^3NeuAcnLc_6$ decreased. This suggests activation of the biosynthetic pathway of a-series gangliosides [13] and inhibition of biosynthesis of some neolacto-series gangliosides. An appreciable increase in G_{M3} (2.2-fold) and a decrease in $VI^3NeuAcnLc_6$ were observed before morphological changes of NaBT-treated cells.

3.2. Artificial remodeling of ganglioside pattern and its effect on HCT 116 cells

To explore the biological significance of ganglioside pattern change during the induction of differentiation, we tried to artificially induce such a ganglioside pattern in HCT 116 cells and examine its effect on them. A fungal metabolite, BFA, has been reported to inhibit de novo biosynthesis of neolacto-series glycolipids and to eventually increase G_{M3} [14]. We used BFA to produce the same ganglioside pattern change as that observed early in the NaBT-induced differentiation of HCT 116 cells.

When HCT 116 cells were treated with 0.1 μg/ml (0.36 μM) BFA, we observed a striking increase (6.5-fold) in de novo biosynthesis of G_{M3} and a concomitant decrease in the biosynthesis of other ganglio-series and neolacto-series gangliosides after 6 h of treatment (Fig. 2A). This eventually gave rise to a ganglioside pattern in BFA-treated cells, in which G_{M3} increased 5.2-fold (Fig. 2C). Following the change in de novo biosynthesis of gangliosides, we observed clear morphological changes to differentiated epithelial cells [15], which included cell flattening, formation of distinct intercellular junctions, and nuclear condensation, with growth suppression in BFA-treated cells after 1 day of treatment (Fig. 2B). The treated cells then gradually became spherical with culture time, suggesting changes toward apoptosis. Flow cytometric analysis

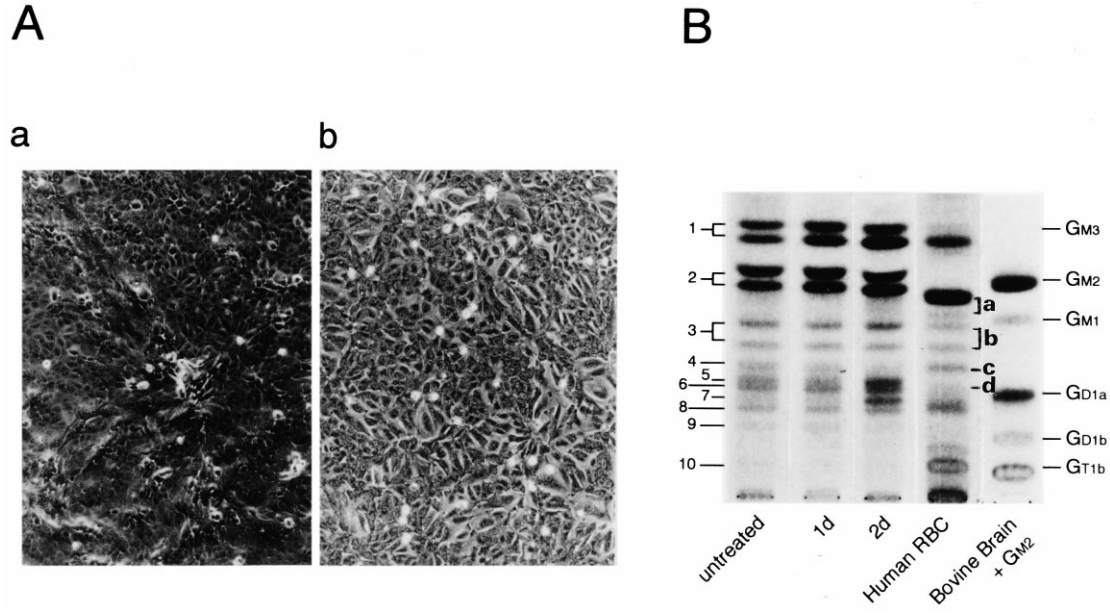

Fig. 1. Differentiation-related changes in ganglioside pattern in HCT 116 cells. Differentiation of HCT 116 cells was induced with 1 mM NaBT. A: Morphological changes after 2 days of treatment. A confluent culture was examined by phase-contrast light microscopy (magnification, ×90). B: Ganglioside profiles of treated cells. Ganglioside fractions corresponding to 5×10^6 cells were applied. Gangliosides of human red blood cells (RBC) and a mixture of bovine brain gangliosides and G_{M2} were used as standards for neolacto-series gangliosides and ganglio-series gangliosides respectively. A doublet band a represents sialosyl α2→3-lacto-*neo*tetraosylceramide; a doublet band b represents sialosyl α2→6-lacto-*neo*tetraosylceramide; band c, sialosyl α2→3-lacto-*nor*hexaosylceramide; band d, sialosyl-α2→6-lacto-*nor*hexaosylceramide [25,26]. After development, the HPTLC plate was sprayed with orcinol-H_2SO_4 reagent, and bands were made visible by heating.

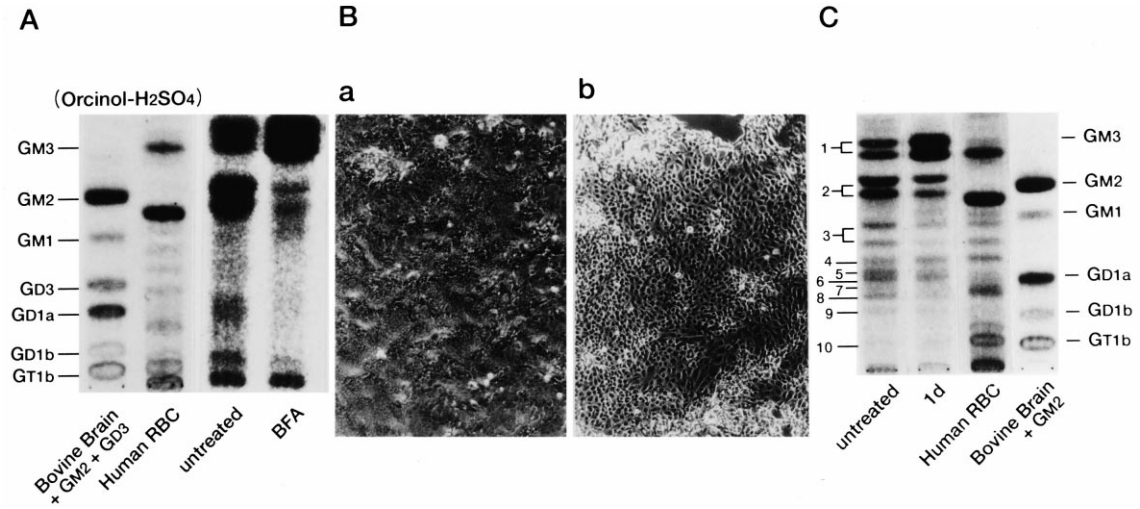

Fig. 2. Induction of differentiation in HCT 116 cells by ganglioside remodeling. HCT 116 cells were treated with 0.1 μg/ml BFA. A: Changes in de novo biosynthesis of gangliosides after 6 h of treatment. Ganglioside fractions corresponding to 1×10^7 cells were applied. Bands were made visible by a BAS 2000 imaging analyzer. B: Morphological changes in treated cells under phase-contrast light microscopy (magnification, ×90). BFA-treated cells formed a typical epithelial sheet with bright cellular contact after 1 day of treatment. C: Ganglioside profiles of treated cells. Ganglioside fractions corresponding to 5×10^6 cells were applied. After development, the HPTLC plate was sprayed with orcinol-H_2SO_4 reagent, and bands were made visible by heating.

showed that BFA-treated cells were arrested in the G_0/G_1 phase (79.5% of cells in the G_0/G_1 phase, 6.0% in S, and 14.5% in G_2/M versus 65.1%, 18.6%, and 16.3%, in control culture after 1 day of treatment), and then the cells gradually initiated apoptosis (Fig. 3). Fragmentation of DNA, which is a marker for apoptosis, was observed after 3 days of treatment (Fig. 4A).

No apparent differentiation-inducing activity of BFA was observed at 0.02 μg/ml, and cytotoxic effects of BFA appeared at 0.2 μg/ml.

3.3. *Effect of forskolin on differentiation-inducing activity of BFA*

BFA has been reported to disrupt the Golgi apparatus and to subsequently inhibit protein secretion [16]. To investigate the implications of this in the molecular mechanism of BFA-induced differentiation of HCT 116 cells, we examined the effect of forskolin, which has been shown to inhibit and reverse the effects of BFA on the Golgi apparatus and to reverse BFA's block on protein secretion [17], on the differentiation-inducing activity of BFA. Forskolin (50 μM) did not affect the BFA-induced change in ganglioside pattern (data not shown), morphological differentiation, or DNA fragmentation (Fig. 4B). This suggests that the effects of BFA on the Golgi apparatus and protein secretion are independent of its differentiation-inducing activity.

4. Discussion

We have shown that human epithelial carcinoma cells can be induced to differentiate into terminally differentiated cells by remodeling the ganglioside pattern by using BFA, which affects ganglioside biosynthesis, suggesting that ganglioside remodeling could be a potent target for differentiation therapy of cancer. Functional differentiation in BFA-treated cells has not yet been examined. However, even if functional differen-

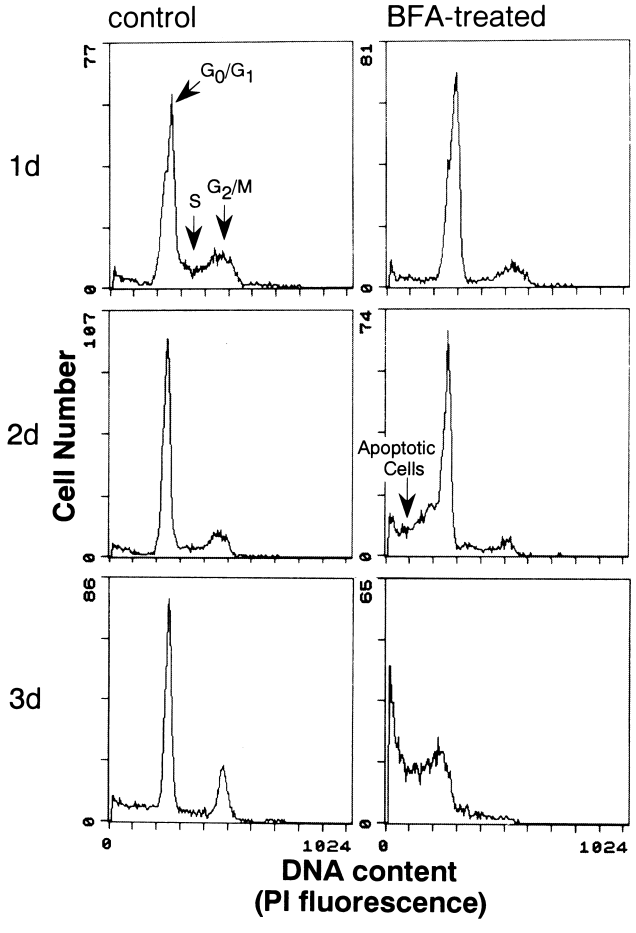

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Fig. 3. Cell cycle analysis of BFA-treated HCT 116 cells. Cells were cultured in the absence or presence of 0.1 μg/ml BFA for the indicated periods of time. The cells were fixed, stained with PI, and analyzed by cytofluorometry.

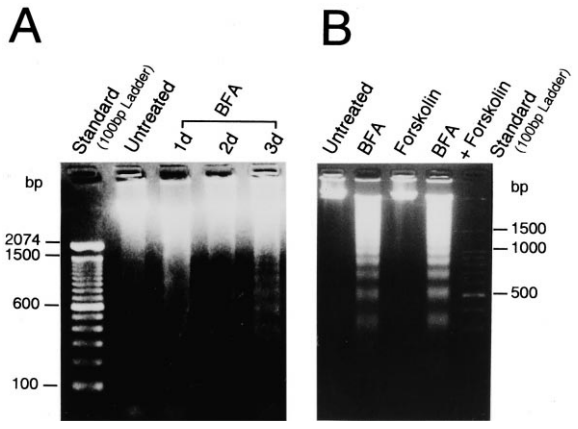

Fig. 4. DNA fragmentation induced in BFA-treated HCT 116 cells. A: Cells were treated with 0.1 μg/ml BFA for the indicated periods of time. B: Cells were treated for 3 days with 0.1 μg/ml BFA in the presence of 50 μM forskolin, which antagonizes the effects of BFA on protein transport and the Golgi apparatus.

tiation is induced in cancer cells, the cells do not necessarily lose their malignant phenotypes [18,19]. It seems to be very important in cancer cell differentiation to induce immortalized cancer cells to die a natural death. Therefore, we assessed terminal differentiation of cancer cells by apoptosis that is induced after morphological differentiation of the cells.

In a wide variety of mammalian cells, BFA has been reported to inhibit protein transport beyond the endoplasmic reticulum (ER)-Golgi system and to cause disassembly of the Golgi apparatus and its mixing with the ER, but not to affect the *trans*-Golgi network [16]. However, it is considered that BFA's induction of differentiation exists not for these activities but for ganglioside pattern changes, especially increase in G_{M3}, for two reasons: neither the effect of BFA on ganglioside biosynthesis nor the terminal differentiation-inducing activity of BFA was inhibited by forskolin, and exogenously added G_{M3} also induced terminal differentiation in HCT 116 cells (H. Nojiri, unpublished results). It was suggested that differentiation of human colonic carcinoma cells is also promoted by specific ganglioside. Forskolin did not affect BFA-induced ganglioside changes in our study, which suggests that membrane traffic between Golgi stacks remained inhibited after morphological recovery of the Golgi apparatus.

NaBT has been reported to elevate CMP-sialic acid:lactosylceramide sialyltransferase (G_{M3} synthase) activity, and to increase G_{M3} levels in human epithelial carcinoma cells [18,20]. This suggests that NaBT might exert its differentiation-inducing activity by increasing G_{M3} levels. An appreciable increase in G_{M3} was also observed before morphological changes both in NaBT-treated HCT 116 cells and in BFA-treated cells. As there is a threshold concentration of ganglioside for induction of differentiation [3,4], an NaBT-induced increase of G_{M3} in HCT 116 cells might not be enough for induction of sufficient differentiation. Other well-known differentiation inducers such as dimethylsulfoxide, retinoic acid, and 12-*O*-tetradecanoylphorbol 13-acetate also increase gangliosides that have differentiation-inducing activity [2–4]. Some differentiation inducers might exert their activities via induction of gangliosides that have differentiation-inducing activity. Conversely, drugs that can induce such bioactive gangliosides could be differentiation inducers.

It has been reported that although the mechanism of antitumor activity of BFA remains unknown, BFA exhibits stronger antitumor activity in vitro against a variety of leukemia and solid tumor cell lines of human origin than against those of murine origin [21]. This antitumor effect might be due to induction of apoptosis (terminal differentiation) by BFA-induced ganglioside change, and the selectiveness might be due to differences in susceptibility of the cells to the ganglioside change. Because ganglioside change in differentiation of human cancer cell is different from that in murine cancer cells [2,22], gangliosides that induce terminal differentiation in human cancer cells might therefore not necessarily induce differentiation in murine cancer cells.

In addition to increasing G_{M3}, BFA inhibits biosynthesis of neolacto-series glycolipid core chains [11]. This specificity of BFA seems to be very important, because neolacto-series glycolipids include gangliosides with sialyl Le^x and sialyl Le^a antigens, which are ligands for E-selectin and are closely related to cancer metastasis [23]. Furthermore, neolacto-series gangliosides accumulate in human colonic adenocarcinoma and are either low or not found in normal colonic mucosa [6,24]. Inhibition of biosynthesis of neolacto-series gangliosides might also be significant for induction of differentiation in human colonic carcinoma cells. Studies focusing on the molecular mechanism by which specific gangliosides induce cancer cell differentiation are now in progress.

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